PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number:	WO 92/08798
C12N 15/82, 5/10, A01H 5/00	A1	(43) International Publication Date:	29 May 1992 (29.05.92)

(21) International Application Number: PCT/GB91/01956

(22) International Filing Date: 7 November 1991 (07.11.91)

(30) Priority data:
9024323.9 8 November 1990 (08.11.90) GB

(71) Applicant *(for all designated States except US)*: IMPERIAL CHEMICAL INDUSTRIES PLC [GB/GB]; Imperial Chemical House, Millbank, London SW1P 3JF (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : BIRD, Colin, Roger [GB/GB]; 31 Fairfax, Bracknell, Berkshire RG12 1YT (GB). GRIERSON, Donald [GB/GB]; 6 Tyler Court, Shepshed, Loughbrough LE12 9SJ (GB). SCHUCH, Wolfgang, Walter [DE/GB]; 14 Greenfinch Close, Heathlake Park, Crowthorne, Berkshire RG11 6TZ (GB).

(74) Agent: ROBERTS, Timothy, Wace; Imperial Chemical Industries plc, Legal Dept., Patents, P.O. Box N°6, Bessemer Road, Welwyn Garden City, Herts AL7 1HD (GB).

(81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE, DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL (European patent), NO, PL, RO, SD, SE (European patent), SN (OAPI patent), SU+,TD (OAPI patent), TG (OAPI patent), US.

Published
With international search report.

(54) Title: EXPRESSION OF GENES IN TRANSGENIC PLANTS

(57) Abstract

DNA construct for use in transforming plant cells comprises an exogenous gene with upstream promoter and downstream terminator sequences, the promoter being a DNA sequence of not less than about 5 kilobases homologous to the DNA control sequence found upstream of the tomato PG gene. Preferably the terminator is homologous to the DNA control sequence of about 1.6 kilobases found downstream of the tomato polygalacturonase gene. Enhanced expression of the exogenous gene is obtained. The invention also includes plant cells containing such constructs and plants derived therefrom.

* See back of page

+ DESIGNATIONS OF "SU"

Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU+	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

EXPRESSION OF GENES IN TRANSGENIC PLANTS

The present invention relates to the expression of genes in transgenic plants. In particular it describes the isolation and use of DNA sequences which permit a high level of expression of foreign genes in transgenic plants.

The ability to isolate and manipulate plant genes has opened the way to gain understanding about the mechanisms involved in the regulation of plant gene expression. This knowledge is important for the exploitation of genetic engineering techniques, applied to problems such as the expression of genes in genetically manipulated crop plants. A large number of examples are now in the literature of plant DNA sequences which have been used to drive the expression of foreign genes in plants. In most instances the regions immediately 5' to the coding regions of genes have been used in gene constructs. These regions are referred to as promoter sequences. They may be derived from plant DNA; or from other sources, e.g., viruses. It has been demonstrated that sequences up to 500-800 bases in most instances are sufficient to allow for the regulated expression of foreign genes. This regulation has involved tissue-specificity; regulation by external factors such as light, heat treatment, chemicals and hormones; and developmental regulation.

These experiments have been carried out using gene fusions between the promoter sequences and foreign genes such as bacterial promoter genes, etc.

Although regulation has been observed this has been hampered by two factors:

1. The low level of expression observed for the transgene in comparison with the endogenous gene. In most instances expression of the transgene has been approximately 1-10% of the expression achieved when the same promoter drives the endogenous gene. This has led to the suggestion that sequences internal to genes may also be important for efficient expression. This has been supported by experiments in which complete genes including 5' and 3' regions as well as coding regions have been used in blot transformation experiments. The influence of sequences surrounding the introduced transgene on the level of expression which can be achieved is normally referred to as 'position effect'. For practical purposes it is desirable that gene constructs introduced into plants give expression levels comparable with that of an endogenous gene. In practice, promoters may be chosen for gene constructs because of their induction pattern, e.g. their tissue specificity or temporal pattern of expression. However, the level of expression of the transgene is usually critical; if the desired promoter cannot give a high enough level of expression it will not be useful.

2. Great variation exists in the level of expression of transgenes between different transformed plant lines. It is not clear why this should be so: it may be another manifestation of the "position effect". These expression levels can differ by as much as two orders of magnitude. Thus a large number of transformants may need to be analysed before one exhibiting the desired expression level can be identified.

These two factors make it very costly and time-consuming to use known promoter constructs for practical genetic plant engineering.

The object of the present invention is to provide novel plant gene constructs which when used to transform plant cells give a high and reliable expression of the inserted gene.

According to the present invention we provide a DNA construct for use in transforming plant cells which comprises an exogenous gene under the control of upstream promoter and downstream terminator sequences, characterised in that the upstream promoter is a DNA sequence of not less than about 5 kilobases that is homologous to the DNA control sequence found upstream of the tomato polygalacturonase gene. Preferably that the downstream terminator is a DNA sequence that is homologous to the DNA control sequence of about 1.6 kilobases found downstream of the tomato polygalacturonase gene. We further provide plant cells transformed with such constructs, and plants containing or composed of such cells.

By the term 'exogenous gene' we indicate a stretch of DNA adapted to be transcribed into functional RNA under the action of plant cell enzymes such as RNA polymerase. Functional RNA is RNA which affects the biochemistry of the cell: it may for example be mRNA which is translated into protein by ribosomes; or antisense RNA which inhibits the translation of mRNA complementary to it into protein. In principle all kinds of exogenous genes are useful in the present invention. The gene may for example be a marker gene such as gus; a gene coding for an insecticidal protein such as the δ-endotoxin from Bacillus thuringiensis; or a gene conferring herbicide resistance, e.g. to glyphosate or to a herbicide inhibiting the ALS pathway. A very wide variety of functional exogenous genes is known from the literature, and the present invention is applicable to these as well as to many others. As well as

functional genes, the exogenous gene may code for other types of functional RNA: for example antisense RNA complementary to any kind of mRNA produced by the plant cell: for example, antisense RNA complementary to mRNA from fruit ripening genes such as polygalacturonase. It is also found that the expression or translation of full-length mRNA of certain genes can be interfered with by expression of truncated RNAs ('sense RNA") having the same sequence as part of the gene. The present invention may be used to generate such 'sense RNA' and thereby downregulate

Plant cells according to the invention may be transformed with constructs of the invention according to a variety of known methods (Agrobacterium Ti plasmids, electroporation, microinjection, etc.). The transformed cells may then in suitable cases be regenerated into whole plants in which the new nuclear material is stably incorporated into the genome. Both transformed monocot and dicot plants may be obtained in this way, although the latter are usually more easy to regenerate.

In work leading to the present invention we have found that sequences upstream of the polygalacturonase (PG) gene permit the regular expression of foreign genes in tomato plants at a high level. We have previously described the isolation of overlapping clones of tomato DNA isolated from a genomic library established in EMBL3 bacteriophage. We have also disclosed the isolation of a 1.45 KG promoter fragment which gives fruit-specific and ripening-specific expression of foreign genes in tomato (Bird et al, Plant Mol. Biol. 11, pp 651-662, 1988). We have now constructed a series of additional vectors, one of which has given high levels of expression of a foreign gene in tomato fruit. The expression levels obtained are of the same order as those obtained for the endogenous PG gene. This represents a major improvement over expression levels obtained previously.

The invention will be further described with reference to the drawings, in which:

Figure 1 is a diagram of DNA vector pCB13;
Figure 2 is a diagram of DNA vector pCB17;
Figure 3 is a diagram of DNA vector pCB19.

The following Examples illustrate the invention.

EXAMPLE 1

A. Construction of pCB13.

The PG (polygalacturonase) promoter region in plasmid pCB1 (Bird et al Plant Molecular Biology 11, 651-662, 1988) was extended by the addition of a 3.5 KG fragment from genomic clone gTOM23 (deposited on 5 December 1986 at the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland, NCIMB Accession Number 12373). The 7.3 KB SalI/BamHI fragment adjacent to the left arm of lambda EMBL3 in gTOM23 was cloned into the SalI/BamHI sites of pUC8 to give plasmid pGTOM23.7.3. The 3.5 kb HindIII fragment from pGTOM23.7.3 was isolated and cloned into the HindIII site of pCB1. Plasmids with the correct orientation of the 3.5 kb HindIII insert contained a 4.9 kb SalI/BamI fragment. One such clone was designated pCB13 (Figure 1).

B. Construction of pCB17.

A 1.6 kb region from the 3' end of the tomato PG gene was substituted for the nopaline synthase polyadenylation sequence in pCB1 (Bird et al Plant Molecular Biology 11, 651-662, 1988).

The 5.8 kb SalI/BamHI fragment adjacent to the right arm of lambda EMBL3 in gTOM23 was cloned into the SalI/BamHI sites of pUC8 to give plasmid pGTOM23.5.8. The 1.6 kb BgIII fragment from pGTOM23.5.8 was isolated and cloned into the BamHI site of pUC19. Plasmids with the correct orientation of the 1.6 kb BgIII insert contained a 550 bp XbaI/BstEII fragment. One such clone was designated A3/1.

A 2.2 kb HindIII/PvuI was isolated as a fragment from pCB1. This contained a 1.45kb PG promoter fragment and the chloramphenicol acetyl transferase (CAT) gene. This was cloned into Bin19 (Bevan, Nucleic Acids Research, 1984, 12, 8711-8721) which had been cut with SalI followed by filling of the cohesive ends with T4 DNA polymerase and subsequently digested with HindIII. Plasmids with the 2.2 kb HindIII/PvuI fragment contained a 2.2 kb HindIII/XbaI fragment. One of these clones was digested with XbaI and KpnI and ligated with the 1.6 kb XbaI/KpnI fragment from A3/1. After transformation, one clone with the correct insert was designated pCB17 (Figure 2).

The correct construction of pCB17 was checked by nucleotide sequence analysis of the plasmid DNA at the boundary between the CAT gene and the PG 3' fragment. An unexpected region of the Bin19 polylinker was found to have remained at this junction. This was judged to be unlikely to interfere with the correct functioning of the plasmid. The sequence of pCB17 in this region is:

XbaI

...CCGTCCCCGTGCATGCCTGCAGGTCGACTCTAGAGGATCTTCAATATATAG...
CAT BIN19 POLYLINKER PG 3'

C. Construction of pCB19

The PG promoter region in plasmid pCB17 was extended by the addition of a 3.5 kb fragment from genomic clone gTOM23 (NCIMB Accession Number 12373).

The 3.5 kb HindIII fragment from pGTOM23.7.8 was cloned into the HindIII site in pCB17. Plasmids with the correct orientation of the 3.5 kb Hind AYE insert contained a 4.9 kb SaII/BamI fragment. One such clone was designated pCB19 (Figure 3). This is a construct according to the present invention.

EXAMPLE 2

Generation of transformed plants

The vectors pCB13, pCB17 and pCB19 were transferred to Agrobacterium tumefaciens LBA 4404 (a micro-organism widely available to plant biotechnologists) and were used to transform tomato plants. Transformation of tomato stem segments followed standard protocols (e.g., (Bird et al Plant Molecular Biology 11, 651-662, 1988). Transformed plants were identified by their ability to grow on media containing the antibiotic kanamycin. Plants were regenerated and grown to maturity. Ripening fruit were analysed for expression of the CAT gene (Bird et al Plant Molecular Biology 11, 651-662, 1988).

Table 1 shows the results of a comparison of plants transformed with the previously defined PG promoter (Bird et al., Plant Molecular Biology 11, 651-662, 1988) and plants transformed with the newly described vectors.

TABLE 1

CAT Expression In Transgenic Plants

VECTOR	PROMOTER	3' END	CAT units/mg protein
pCB1	1.45 Kb	nos	0.66
pCB13	5 Kb	nos	0
pCB17	1.45 Kb	1.8 kb PG	0.85
pCB19	5 Kb	1.8 Kb PG	1456

Measurements of CAT and PG mRNA indicated that the level of CAT protein expression is of the same order of magnitude as PG expression.

We claim:

1. DNA construct for use in transforming plant cells which comprises an exogenous gene under the control of upstream promoter and downstream terminator sequences, characterised in that the upstream promoter is a DNA sequence of not less than about 5 kilobases that is homologous to the DNA control sequence found upstream of the tomato polygalacturonase gene.

2. Construct as claimed in claim 1 in which the downstream terminator sequence is homologous to the DNA control sequence of about 1.6 kilobases found downstream of the tomato polygalacturonase gene.

3. Construct as claimed in either of claims 1 or 2 in which the exogenous gene codes for an insecticidal protein.

4. Construct as claimed in either of claims 1 or 2 in which the exogenous gene generates antisense RNA.

5. A plant cell comprising a DNA construct claimed in any of claims 1 to 4 and adapted to transcribe RNA from the exogenous gene.

6. A plant cell as claimed in claim 5 in which the RNA transcribed is mRNA which is subsequently translated by the cell into protein.

7. A plant cell as claimed in claim 5 in which the RNA transcribed inhibits production of a protein produced by the cell.

8. A plant cell as claimed in claim 7 in which the RNA transcribed is antisense to the mRNA of the protein of which production is inhibited.

9. Plants comprising cells claimed in any of claims 5 to 8; and seeds and progeny thereof.

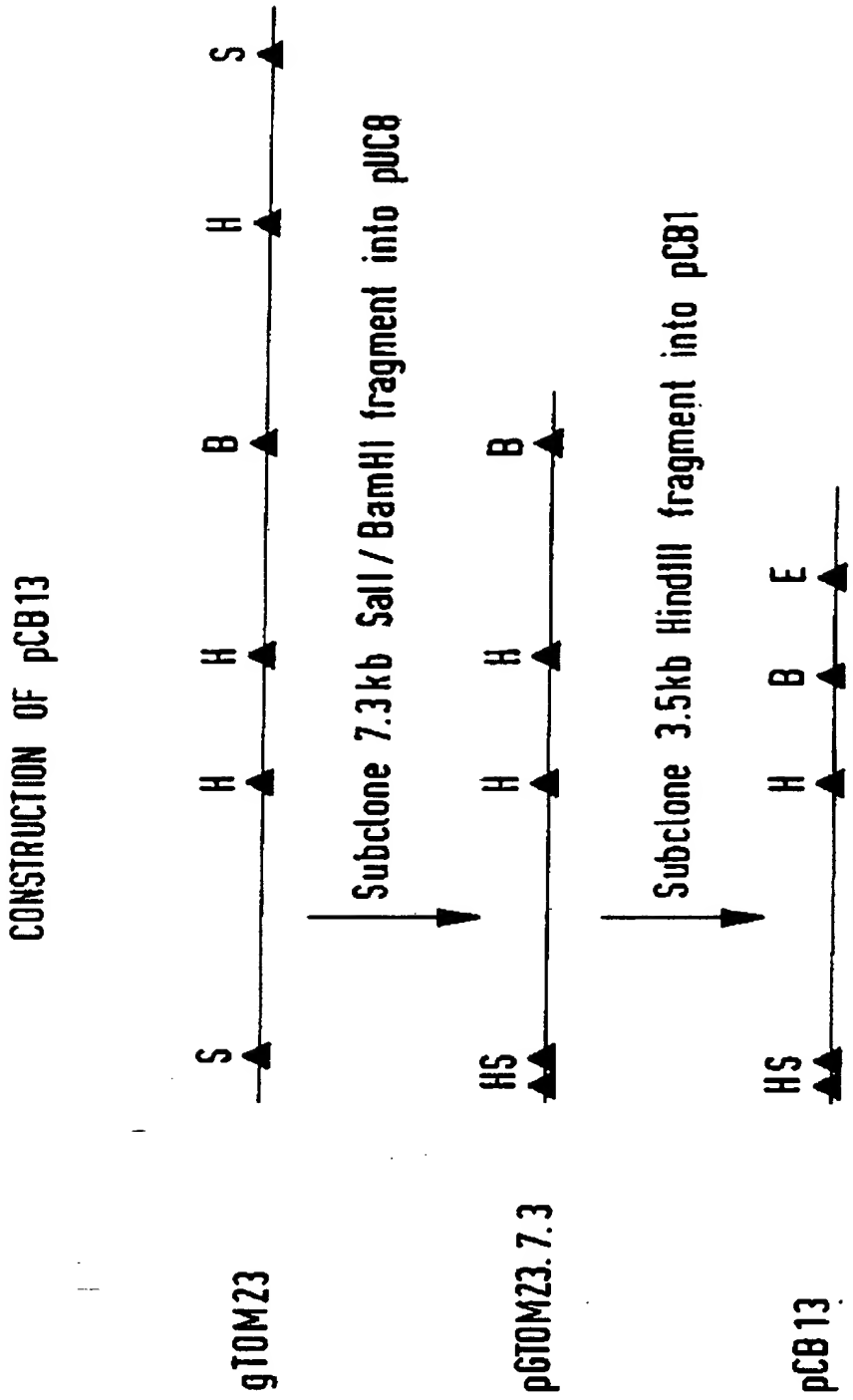
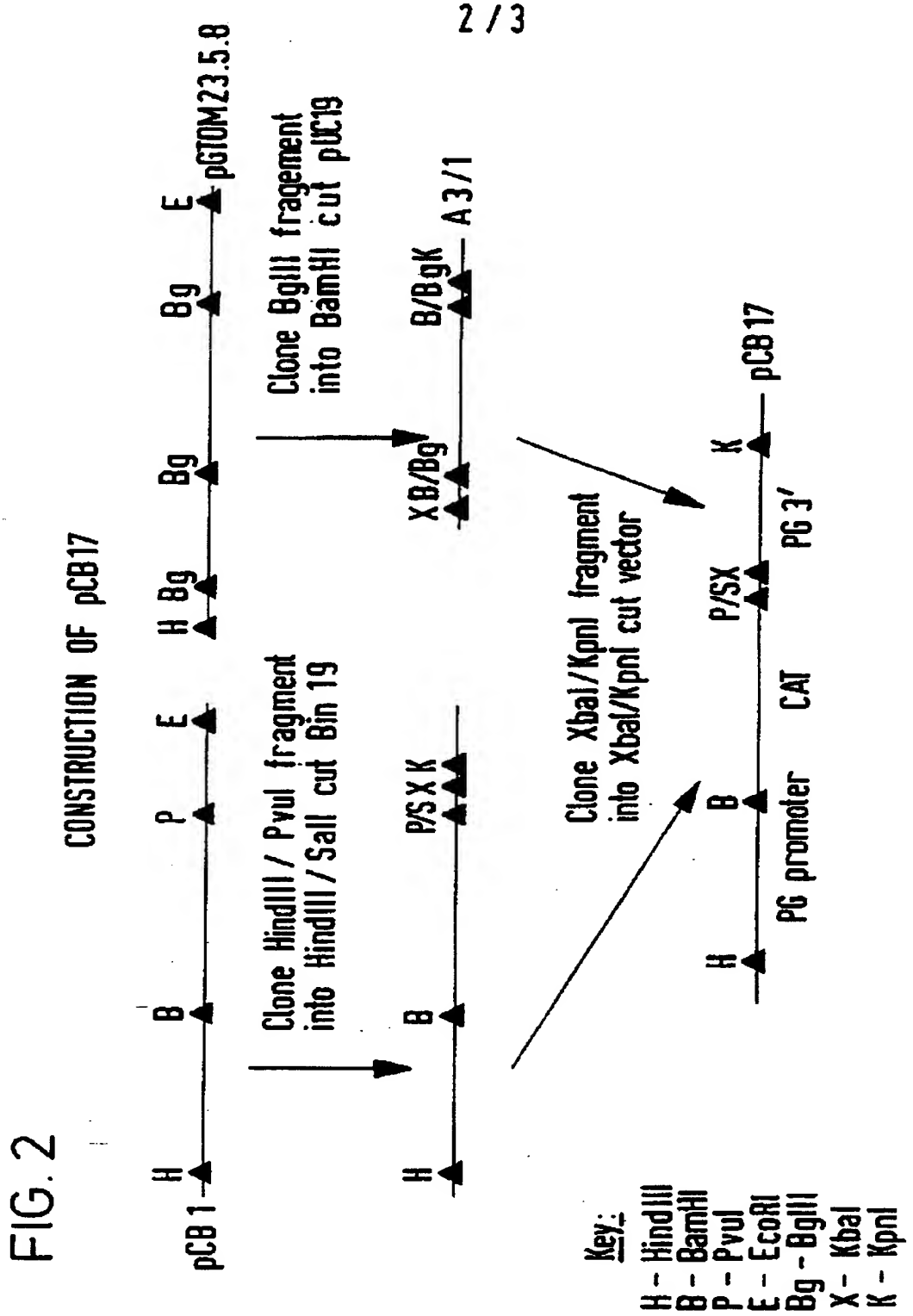


FIG. 1

FIG. 2



SUBSTITUTE SHEET

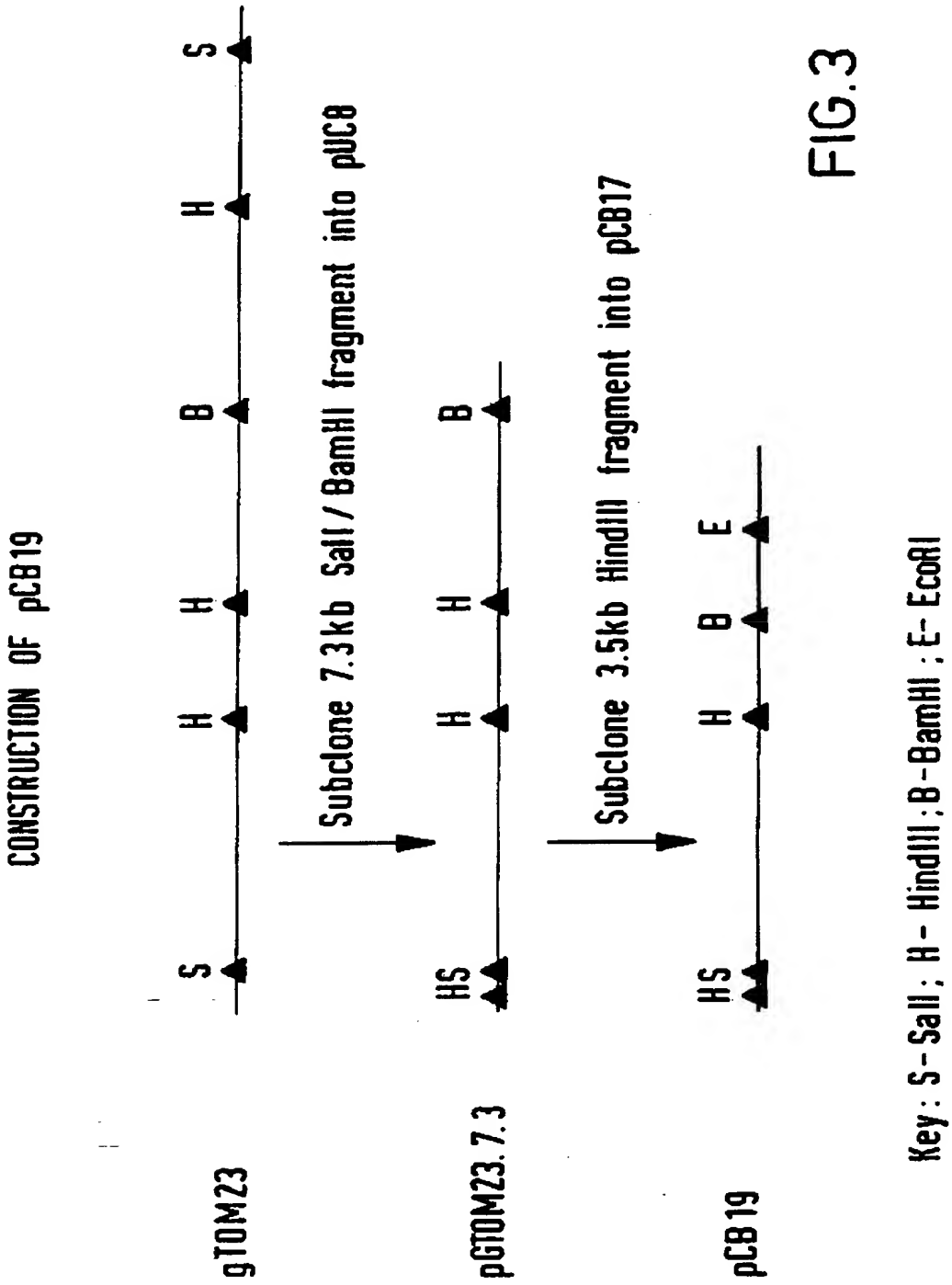


FIG.3

INTERNATIONAL SEARCH REPORT

PCT/GB 91/01956

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/82; C12N5/10; A01H5/00

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A01H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	WO,A,9 109 128 (ICI) 27 June 1991 see examples 6,7	1,2,4-9
A	EP,A,0 271 988 (ICI) 22 June 1988 see the whole document	1-9
A	WO,A,8 912 386 (CALGENE) 28 December 1989 see page 39, line 17 - page 41, line 17	1-9
A	PLANT MOLECULAR BIOLOGY. vol. 11, 1988, DORDRECHT, THE NETHERLANDS. pages 651 - 662; BIRD, C. R., ET AL.: 'The tomato polygalacturonase gene and ripening - specific expression in transgenic plants' see the whole document	1-9

-/—

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
06 FEBRUARY 1992	13.02.92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MADDOX A.D.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	BIOLOGICAL ABSTRACTS BR39:65917 see the abstract & J. EXP. BOTANY vol. 41, 1990, P5-6 SHABBEER, J., ET AL.: 'Putative regulatory factors binding a fruit ripening promoter'	1-9

Form PCT/ISA/210 (extra sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. GB 9101956
SA 53067

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 06/02/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9109128	27-06-91	AU-A- 6893891	18-07-91
EP-A-0271988	22-06-88	AU-A- 7435091	11-07-91
		AU-A- 8095687	12-05-88
		JP-A- 63164892	08-07-88
		US-A- 5073676	17-12-91
WO-A-8912386	28-12-89	AU-A- 3852089	12-01-90

EPO FORM P079

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82